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Inactive Matriptase-2 Mutants Found in IRIDA Patients Still Repress Heparin in a Transfection Assay Despite Having Lost Their Serine Protease Activity

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Abstract

Mutations of the *TMPRSS6* gene, which encodes Matriptase-2, are responsible for Iron Refractory Iron-Deficiency Anemia. Matriptase-2 is a transmembrane protease that down-regulates hepcidin expression. We report one frameshift (p.Ala605ProfsX8) and four novel missense mutations (p.Glu114Lys, p.Leu235Pro, p.Tyr418Cys, p.Pro765Ala) found in IRIDA patients. These mutations lead to changes in both the catalytic and non-catalytic domains of Matriptase-2. Analyses of the mutant proteins revealed a reduction of autoactivating cleavage and the loss of N-Boc-Gln-Ala-Arg-p-nitroanilide hydrolysis. This resulted either from a direct modification of the active site or from the lack of the autocatalytic cleavage that transforms the zymogen into an active protease. In a previously described transfection assay measuring the ability of Matriptase-2 to repress the hepcidin gene (*HAMP*) promoter, all mutants retained some, if not all, of their transcriptional repression activity. This suggests that caution is called for in interpreting the repression assay in assessing the functional relevance of Matriptase-2 substitutions. We propose that Matriptase-2 activity should be measured directly in the cell medium of transfected cells using the chromogenic substrate. This simple test can be used to

determine whether a sequence variation leading to an amino acid substitution is functionally relevant or not.

Key words: *TMPRSS6*, Matriptase-2, hepcidin, anemia, IRIDA

Introduction

Patients with Iron Refractory Iron Deficiency Anemia (IRIDA; MIM# 206200) absorb insufficient amounts of iron from the diet and respond inadequately to oral iron therapy. IRIDA is characterized by low serum iron, low serum ferritin, and high plasma levels of hepcidin, the master regulator of systemic iron homeostasis. This disease is caused by biallelic mutations of the *TMPRSS6* gene (MIM# 609862) that encodes Matriptase-2 (MT2), a transmembrane serine protease of the type-two transmembrane serine protease (TTSP) family, which is mainly expressed in the liver. The role of MT2 in iron homeostasis was initially demonstrated by the discovery of a homozygous splice site mutation of *Tmprss6* in the Mask mouse (Du, et al., 2008) followed by the characterization of the *Tmprss6* knockout mice (Folgueras et al., 2008). These mice suffer from microcytic anemia due to reduced absorption of dietary iron caused by high levels of hepcidin. Hepcidin gene (*HAMP*) expression is mainly dependent upon Bone Morphogenetic Protein 6 (BMP6) and hemojuvelin (HJV). Binding of the BMP6 cytokine to its receptors activates a signaling cascade leading to *HAMP* transcription via phosphorylation of Son of Mother Against Decapentaplegic (SMAD) 1/5/8 effectors. HJV, a GPI-linked membrane protein synthesized by the hepatocytes, is a BMP6 coreceptor (Babitt, et al., 2006; Xia, et al., 2008). The critical role of the BMP6/HJV/SMAD pathway in iron homeostasis is supported by

the loss of hepcidin expression and massive parenchymal iron overload observed in several mouse models, including mice in which BMP type I receptor genes (*Alk2*, *Alk3*) have been deleted in the liver (Steinbicker, et al., 2011), *Bmp6*^{-/-} (Meynard, et al., 2009, Andriopoulos, et al., 2009) and *Hjv*^{-/-} mice (Vaulont, et al., 2005) as well as in mice with targeted liver deletion of *Smad4* (Wang, et al., 2005). Similar severe iron overload (juvenile hemochromatosis) is observed in patients with mutations in *HFE2*, which encodes for the HJV protein. On the basis of transfection experiments, it has been postulated that MT2 down-regulates hepcidin levels by binding to and proteolytically degrading the BMP co-receptor HJV (Silvestri, et al., 2008). However, recent experiments performed *in vivo* do not support this hypothesis. Indeed, Gibert et al showed that the increase in hepcidin expression that follows the knockdown of the *Tmprss6* gene in a zebrafish embryo is independent of HJV (Gibert, et al., 2011). Furthermore, Krijt et al showed that in *Tmprss6*^{-/-} mice, the amount of membrane-bound HJV (mHJV) protein in liver was significantly reduced, and not increased as expected (Krijt, et al., 2011).

From its N- to its C-terminus, MT2 is composed of a small cytoplasmic domain, a transmembrane domain, a stem region consisting of a SEA (sea urchin Sperm protein Enterokinase Agrin) domain, two CUB (C1s/C1r, Urchin embryonic growth factor and Bone morphogenetic protein) domains, three LDLRA (low-density lipoprotein receptor class A) domains, and a carboxy terminal serine protease (SP) domain (see Figure 1A). Three residues in the SP domain, Histidine 617, Aspartate 668 and Serine 762 (the catalytic triad) are essential for protease activity (Szabo and Bugge, 2011). Like other TTSPs, MT2 is synthesized as a zymogen that needs its own trypsin-like serine protease activity to become activated. Autoactivation of the zymogen is characterized by a cleavage after the Arginine 576 within a highly-conserved Arg-Ile-Val-Gly-Gly (RIVGG) motif located at the junction between the SP domain and the stem

(Figure 1A). It has been shown that MT2 is activated via a transactivation mechanism involving multimerization of the zymogen (Stirnberg, et al., 2010) at the plasma membrane. Following activation, the SP domain remains bound to the stem by a disulfide bond. The active protease is released from the cell membrane into the extracellular medium by proteolytic cleavage within the stem (Stirnberg, et al., 2010). So far, 34 MT2 mutations have been identified in human patients with IRIDA (Altamura, et al., 2010; Beutler, et al., 2009; Choi, et al., 2011; De Falco, et al., 2010; Edison, et al., 2009; Finberg, et al., 2008; Guillem, et al., 2008; Melis, et al., 2008; Ramsay, et al., 2009b; Sato, et al., 2011; Silvestri, et al., 2009; Tchou, et al., 2009). These include missense, nonsense, frameshift, and splice junction mutations. Missense mutations have been found in several different protein domains of the extracellular part of the zymogen, and some of them have been further characterized by transfection experiments, usually by assessing the ability of mutated proteins to repress the hepcidin promoter linked to a luciferase reporter gene (Altamura, et al., 2010; De Falco, et al., 2010; Du, et al., 2008; Ramsay, et al., 2009b; Silvestri, et al., 2009; Silvestri, et al., 2008). Here we describe five mutations observed in IRIDA patients, one homozygous frameshift mutation, found in one family, and four missense mutations located in the CUB1, CUB2, SEA, and SP domains of MT2, found in two families with compound heterozygosity of the affected children. We studied the functional consequences of the mutations on the activation of the zymogen, the release of the protease activity into the medium of transfected cells and the repression of the hepcidin promoter. We also studied two designed mutant controls: p.Arg576Ala (R576A) and p.Ser762Ala (S762A). The (R576A) mutant was designed to prevent the autoactivation of MT2 by replacing the arginine that is targeted by the autocatalytic activating cleavage. The S762A mutant is a catalytically inactive

protein in which the serine residue directly involved in the proteolytic activity of the enzyme has been replaced by an alanine (Stirnberg, et al., 2010).

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Materials and Methods

Patients (see Table 1 and Figure 2)

Family A: Two affected brothers were born to healthy, first-cousin parents from Algeria. The eldest brother received several courses of oral iron during his childhood. No hematological data were available from before he was diagnosed with microcytic anemia when he was 15 years old. At the time of diagnosis he had 7.3 g/dL hemoglobin, and a MCV of 54 fL. Since then, he had received several courses of intravenous iron, and when 25 years of age he had a normal hemoglobin level (12 g/dL), although the microcytosis persisted. His serum ferritin had risen from 6 µg/L (when 15 years of age) to 230 µg/L (when 25 years of age). His younger brother was noted by his parents to display pallor at birth. He was diagnosed with microcytic anemia when he was 1 year old (Hb: 7.4 g/dL and MCV: 51 fL). Intravenous (IV) iron was administered from the age of 8 years, and he responded well, with 12 g/dL of hemoglobin and an MCV of 64 fL at the age of 17 years.

Family B: Two affected brothers were born to non-consanguineous parents of French origin. Both patients presented with microcytic hypochromic anemia at 4 years and 6 months of age, respectively. Occult gastrointestinal blood loss and gluten enteropathy were excluded. Hematological and bone marrow examinations ruled out other possible causes of anemia. With oral iron treatment, both patients reached an acceptable hemoglobin level (11 g/dL), but remained microcytic with low serum iron, normal total iron binding capacity (TIBC), and very low transferrin saturation. Both of them displayed a decrease in hemoglobin concentration when iron supplementation was discontinued.

Family C: The patient was a French boy born to non-consanguineous parents. He was diagnosed with hypochromic microcytic anemia when he was 3 years old, and was given oral iron

continuously for 17 months. His hemoglobin concentration progressively rose to 12 g/dL, but then fell back to 10.5 g/dL when the treatment was discontinued. A year and a half later he was given a single dose of intravenous iron. This was followed by an increase in hemoglobin concentration that persisted for 2 years. He was then given oral iron supplementation again. Serum hepcidin concentrations were measured by ELISA (Intrinsic Life Sciences, USA) and are shown in Table 1.

Blood samples for genetic analysis were obtained from the patients or their parents after they had signed informed consent forms in accordance with the requirements of the French Bioethics legislation.

TMPRSS6 sequencing and DNA constructs

Exons and intron-exon junctions of the *TMPRSS6* gene were sequenced as previously described (Guillem, et al., 2008) and compared to the reference sequence NM_153609.2 (GenBank). Mutant alleles are named according to journal guidelines (www.hgvs.org) and have been submitted to the Leiden Open Variation Database (<http://www.lovd.nl/TMPRSS6>). The full-length human MT2 cDNA with a C-terminal FLAG epitope cloned in the pcDNA3.1 vector was kindly provided by Carlos Lopez Otin. Expressing vectors encoding MT2 mutants found in IRIDA patients: p.Glu114Lys (E114K), p.Leu235Pro (L235P), p.Tyr418Cys (Y418C), p.Pro765Ala (P765A), p.Ala605Pro+8fsX (A605fs), and designed mutants Arg576Ala (R576A) and Ser762Ala (S762A), were obtained by mutagenesis of wild-type cDNA using a Quickchange site-directed mutagenesis kit (Stratagene), sequences of the oligonucleotides are available upon request. To generate MT2-V5 expressing vectors, pcDNA wild type (WT) MT2-FLAG was digested using BstEII and XhoI enzymes, and the digested plasmid was purified on agarose gel

and dephosphorylated. An insert containing a V5 tag was made by hybridizing two oligonucleotides phosphorylated at their 5' extremity (sequences are available upon request), and ligation into the previously digested plasmid. In order to introduce the FLAG sequence in front of the stop codon of the A605fs mutant, we used four primers for Site Directed, Ligase-Independent Mutagenesis (SLIM) according to Chiu et al. protocol (Chiu, et al., 2004): PCR was performed with the four primers Rt 5'cccagcggtcagcgatgagggccccccacagatgtgtcg3', Rs 5'gggccccccacagatgtgtcg3', Fs 5'gactacaaggacgacgatgac3', Ft 5'tcatcgctgaccgctggggactacaaggacgacgatgac3' with pcDNA3 WT MT2 as a DNA template. To generate the pcDNA WT *HJV* construct, the full human ORF was amplified from human liver cDNA and inserted into pcDNA3 using TOPO cloning (Invitrogen). The whole cDNA sequence of each construct was verified by sequencing after mutagenesis.

Cell culture

HeLa and Huh7 cells were cultured at 37°C in Dulbecco's modified Eagle's Medium (DMEM) with L-glutamine and 1 g/L glucose, supplemented with antibiotics (penicillin, streptomycin), and 10% heat decomplexed fetal bovine serum, in 95% humidified air and 5% CO₂.

Western blots on cell lysates and concentrated media

HeLa and Huh7 cells seeded in 10-cm diameter dishes and grown up to 50-70% confluence were transiently transfected with Fugene HD reagent (Roche) in optiMEM according to the Manufacturer's instructions. After 24 h, the medium was replaced with 4.5 mL of fresh optiMEM with antibiotics. The medium was collected 24h later, and concentrated using 10-kDa

molecular weight cutoff ultrafiltration membranes (Amicon ultra, Millipore). Cells were lysed in lysis buffer (Cell Signaling Technology) supplemented with an antiprotease cocktail (Roche). Forty μ g of proteins in denaturing and reducing Laemmli buffer were loaded per well of 10% or 12% polyacrylamide gel for cell lysates (CL) and concentrated media (CM), respectively. Proteins were transferred onto an Immobilon-P transfer membrane (Millipore), using an Invitrogen electrophoresis system. After blotting, membranes were blocked overnight at 4°C in 7% milk diluted in TBS-Tween (0.15%). Membranes were incubated with a mouse anti-FLAG M2 monoclonal antibody (Sigma) diluted 1/10000 (CL) or 1/5000 (CM), and then with a sheep secondary anti-mouse antibody diluted 1/3000 (Amersham Bioscience). Anti-actin primary antibody (Sigma) and anti-mouse secondary antibody (Amersham Bioscience) were used at 1/7500 dilution. All the antibodies were incubated for one hour (with the exception of actin: 30 min) at room temperature. Immunoblots were visualized by chemiluminescence using the HRP (horseradish peroxidase) substrate (Millipore).

Immunofluorescence

Huh7 cells grown to 50-70% confluence on a glass coverslip 1.8 cm in diameter were transiently transfected with pcDNA WT MT2 or with constructs expressing the missense mutants. The transfection medium was replaced after 24h with fresh optiMEM, and immunofluorescence labeling was performed 24h later. The dilution of primary rabbit polyclonal anti-FLAG antibody (Sigma) was 1/250, and the dilution of the anti-rabbit secondary antibody labeled with FITC (Invitrogen) was 1/200.

Luciferase assay

Huh7 cells seeded at 50-70% confluence in a 24-well plate were transiently transfected with TK Renilla plasmid encoding *Renilla reniformis* luciferase, and with a construction containing the *Photinus pyralis* luciferase gene controlled by the *HAMP* gene promoter (Patel, et al., 2012). Cells were also co-transfected with the pcDNA HJV-expressing vector and WT or mutated MT2 expressing plasmid, in optiMEM. After 48h, the cells were lysed with passive lysis buffer (Promega), and the luciferase activity was determined according to the Manufacturer's instructions (Dual glo luciferase reporter assay, Promega). The relative luciferase activity was determined as the ratio of the *HAMP* promoter *Photinus pyralis* firefly to *Renilla reniformis* luciferase activity. Experiments were performed in triplicate.

Measurement of MT2 proteolytic activity

Hela cells were transfected with either WT or mutant MT2-expressing constructs as described above. After 24 hours, 50µg of proteins from concentrated media were used to measure the protease activity by monitoring the release of p-nitroanilide from the chromogenic substrate N-(tert-butoxycarbonyl)-Gln-Ala-Arg-p-nitroanilide (400 µM) at a wavelength of 405 nm during an incubation of 20 minutes in Tris/Saline buffer (50 mM Tris, 150 mM NaCl, pH8.0) at 37°C.

Co-Immunoprecipitation assay

Cell lysates obtained as described for the Western blots were incubated with 2 µg of anti-V5 monoclonal antibodies (Invitrogen) or anti-FLAG monoclonal antibodies for one hour at 4°C. BioAdem beads PAG (protein A and G) diluted in lysis buffer were added to the immune complexes and incubated overnight at 4°C while stirring. After washing three times with lysis

buffer, proteins bound to antibodies were eluted with PAG elution buffer. This experiment was performed using Ademtech magnetic devices.

Results

We describe five patients with IRIDA belonging to three families (Table 1 and Figure 2). Two brothers in family A were diagnosed with typical features of IRIDA: microcytic anemia with low serum iron, high concentration of plasma hepcidin, no response to oral iron and only a partial response to intravenous iron therapy. In contrast, in the other two families, the patients (two brothers in family B and a single affected child in family C), had an unusual clinical history since they had responded partially to sustained oral iron therapy. Otherwise, they also showed typical symptoms of IRIDA with persisting microcytosis under therapy. Their plasma hepcidin level was in the normal range though abnormally high when their hemoglobin concentration was taken into account (Ganz, et al., 2008). However, it should be noted that patients from both these families would have been considered to be unresponsive to oral iron according to the usual criteria, since only long-lasting and sustained oral iron therapy was able to maintain even a subnormal hemoglobin concentration, and the hemoglobin level fell when oral iron was discontinued.

We identified four novel and one previously described mutations of the *TMPRSS6* gene in the three families (Figure 2). In family A, a previously reported mutation (Finberg, et al., 2008) was found in the homozygous state in the two brothers born to consanguineous parents, and consisted of a single nucleotide deletion: c.1813delG; p.Ala605ProfsX8 (A605fs). This mutation was predicted to lead either to RNA degradation or to a truncated protein. In family B, the affected brothers were heterozygous for two missense mutations, c.704T>C; p.Leu235Pro (L235P) in the CUB domain 1 and c.1253A>G; p.Tyr418Cys (Y418C) in the CUB domain 2. The father and the

mother were heterozygous for the L235P mutation and the Y418C mutation, respectively. In family C, the proband was heterozygous for two other missense mutations: c.340G>A; p.Glu114Lys (E114K) in the SEA domain and c.2293C>G; p.Pro765Ala (P765A) in the SP domain. The father and the mother were heterozygous for the P765A mutation and the E114K mutation, respectively. The location of the mutations in the different protein domains is shown on Figure 1A. All four amino acid substitutions affect evolutionarily conserved residues (Figure 1B) and were predicted to be damaging by Polyphen2 software (<http://genetics.bwh.harvard.edu/cgi-bin/pph2>).

MT2 missense mutant proteins are targeted to the plasma membrane

In order to find out whether the missense mutations found in IRIDA patients modify the localization of the protein at the cell membrane, Huh7 cells were transiently transfected with the cDNA encoding the WT MT2 and each of the four missense IRIDA mutants with a FLAG-epitope. In non-permeabilized cells, the MT2 mutants were detected at the membrane of Huh7 cells, in a similar way to the WT protein (Supp. Figure S1).

Repression of HJV-induced HAMP promoter activity by MT2 mutants

To assess the impact of MT2 mutations on hepcidin gene activity, we transfected Huh7 cells with a *HAMP* promoter-*Photinus* luciferase reporter vector and a TK-*Renilla* luciferase vector to normalize transfection efficiency between samples. Cells were also co-transfected with the HJV-expressing vector, and either WT or mutant MT2-expressing vectors. We tested the five IRIDA mutants plus two designed mutants as controls: R576A and S762A. WT MT2 repressed HJV-induced luciferase activity 8 fold compared to HJV transfection alone (Figure 3). Surprisingly,

all the mutants repressed *HAMP* promoter-driven luciferase expression, and only some of them (Y418C, L235P, E114K and R576A) were significantly, although moderately, less efficient than WT MT2. The A605fs mutant displayed only weak repressor activity. We performed this experiment three times, and Figure 3 shows the results of one representative experiment. Repression of the hepcidin promoter was also demonstrated under conditions where HuH7 cells were transfected with MT2-expressing vectors in the absence of the HJV-expressing vector both under unstimulated conditions and in the presence of added BMP2 (Supp. Figure S2). This suggests that both the wild-type MT2 and the missense mutants are able to interfere with endogenous HJV when overexpressed in HuH7 cells.

Autocleavage of MT2 mutants

In order to determine the autocatalytic cleavage activity of MT2 mutants, HeLa cells were transfected with plasmid constructs expressing either WT MT2 or each of the MT2 mutants, or combinations of Y418C/L235P and E114K/P765A mutants. The corresponding proteins were detected in cell lysates (CL) with an apparent molecular weight of 120 kDa for both WT MT2 and the missense mutants, corresponding to full length zymogens (Figure 4A, CL). As expected, the truncated mutant that lacks 198 amino acid residues was detected with an apparent molecular weight of 90 kDa (Figure 4A, CL). When the concentrated medium (CM) of HeLa cells transfected with WT MT2-FLAG cDNA was analyzed using sodium dodecyl sulfate (SDS) PolyAcrylamide Gel Electrophoresis (PAGE) under reducing conditions, the hallmark of the autoactivating cleavage of the zymogen was present, i.e. a 30-kDa fragment corresponding to the SP domain (Figure 4A, CM). As expected, the R576A mutant could not be processed at the RIVGG site, and the corresponding 30-kDa fragment was not detected in the cell medium. The

S762A mutant was not cleaved either, confirming that the catalytic activity of the MT2 zymogen itself is necessary for its activating cleavage to occur. Similarly, no 30-kDa fragment was observed for the Y418C, L235P, and E114K mutants, either alone or in combination while transfection of the P765A mutant resulted in a reduction of the intensity of the 30-kDa fragment compared to that found for WT MT2. For the A605fs mutant, which is completely devoid of the SP domain, no cleavage fragment was detected.

In transfected Huh7 cells, no residual autocleavage was observed with any of the mutants studied (Figure 4B, CM).

MT2 missense mutants are not trans-activated by wild-type protein

Stirnberg, et al. demonstrated that a MT2 protein mutated in the catalytic serine S762A can be modified by trans-cleavage at the RIVGG site by its WT counterpart (Stirnberg, et al., 2010). We investigated whether the missense IRIDA variants could be processed in the same way. Huh7 cells were co-transfected with a plasmid expressing either one of the FLAG-labeled mutants and the WT MT2 expressing construct with a V5 epitope. Full length MT2-V5 and the autoactivation fragment were detected with the anti-V5 antibody in the cell lysate and cell medium, respectively (Figure 5, CL and CM). When the S762A MT2-FLAG mutant was co-transfected with WT MT2-V5, a 30-kDa fragment was also detected with the anti-FLAG antibody by Western blot analysis under reducing conditions, in agreement with data previously reported (Stirnberg, et al., 2010). In contrast, none of the four IRIDA mutants was cleaved by WT MT2. This may indicate a conformational defect that either prevents the formation of MT2 oligomers or modifies the accessibility of the RIVGG cleavage site.

MT2 IRIDA mutations do not alter protein-protein interactions

Since IRIDA mutants were not transactivated by the WT MT2 protein in Huh7, we investigated whether this was a consequence of an impaired interaction between WT and the mutated proteins. HeLa cells were transfected with WT MT2-V5 in the presence of either WT or mutant MT2-FLAG. Cell lysates were immunoprecipitated either with anti-V5 or anti-FLAG antibodies, and the immunoprecipitates were analyzed by immunoblotting with anti-V5 and anti-FLAG antibodies (Supp. Figure S3). WT MT2-FLAG was detected in anti-V5 immunoprecipitate, and reciprocally WT MT2-V5 was present in anti-FLAG immunoprecipitate. All missense IRIDA MT2-FLAG mutated proteins co-immunoprecipitated with WT MT2-V5, which suggests that the defect in the transactivation of IRIDA variants by WT MT2 was not due to impaired interaction between the mutated and WT MT2 protein.

Proteolytic activity in the media of cells transfected with WT and mutated-MT2 using a chromogenic substrate

In order to assess the protease activity resulting from the shedding of MT2 into the culture medium of transfected HeLa cells, proteolytic activity in concentrated medium from transfected cells were assayed, using a chromogenic substrate previously used in two different studies (Meynard, et al., 2011; Sisay, et al., 2010). None of the mutants tested showed any detectable protease activity, whereas absorbance increased linearly with incubation time in the WT MT2 (Figure 6).

Discussion

In this paper, we tested the functional consequences of four new missense mutations of *TMPRSS6* gene identified in five IRIDA patients from three families. We used several different assays with two aims: firstly, to find out whether these novel variants were indeed causal mutations and, secondly, to try to further elucidate the functioning of MT2. We initially used a previously described transfection assay that measures the ability of MT2 mutants to repress the expression of a luciferase reporter gene driven by the *HAMP* promoter. One of the four IRIDA mutants (P765A) repressed *HAMP*-driven luciferase expression to a similar extent as the WT construct, and all the mutants tested still produced some degree of transcriptional repression activity, including the negative control S762A mutant in which the serine of the catalytic triad is replaced by an alanine. Although we found these observations surprising, a careful analysis of the literature shows that several missense mutants also have a repressive effect (Table 2). In one case, similar findings had in fact already been reported (Ramsay, et al., 2009b). In other papers, the focus was solely on the difference between mutants and WT MT2, and the residual repressive activity of the mutants had been overlooked (Altamura, et al., 2010; Du, et al., 2008; Ramsay, et al., 2009b; Silvestri, et al., 2009; Silvestri, et al., 2008).

Since the catalytic activity of MT2 is considered to be essential for its function, we tested it in transfection experiments in two different types of cell: HeLa cells and the hepatocyte cell line Huh7. The IRIDA mutants, the inactive S762A mutant, and an additional mutant R567A all displayed a profound defect in protease activity as compared to the WT construct. This abnormality was revealed by a defective cleavage at the RIVGG sequence that links the SP domain to the stem. This cleavage is required for conversion of the inactive zymogen form of membrane MT2 into an active protease, and is catalyzed by a transactivation mechanism that

involves MT2 dimerization or oligomerization (Stirnberg, et al., 2010). This autocatalytic cleavage was severely impaired for all the mutants, except P765A, for which it was partially conserved in HeLa cells, and it was absent for all mutants in Huh7 cells. This observation suggests that this cleavage may somehow depend on the tissue-specific microenvironment of the protein. There was an absence of cleavage at the RIVGG sequence of the four IRIDA mutants by the WT MT2 in cotransfection experiments, whereas the control mutant, S762A, was cleaved by the WT MT2. This suggests that the four missense mutations studied here might induce a change in protein conformation that reduces the accessibility of their cleavage site.

To evaluate the proteolytic activity in the culture medium from HeLa cells expressing MT2 proteins with IRIDA mutations, we used a synthetic chromogenic substrate. Interestingly, none of the four missense mutants, either separately or in combination (Y418C/L235P and E114K/P765A), displayed any detectable proteolytic activity against the substrate in contrast to WT MT2. For the three mutants Y418C, L235P, and E114K, this finding was in agreement with the absence of MT2 SP domain released from the cell membrane. Surprisingly, the P765A mutant showed no activity either, despite a detectable amount of autoactivation fragments in the media (Figure 4A). Noteworthy, amino acids 750 to 810 in the MT2 protein are essential for serine protease function as this region constitutes the substrate binding site (Ramsay, et al., 2009a). As a consequence, the P765A mutation might modify the affinity of the protease for the substrate, most likely explaining its defective serine protease activity when released into the cell culture medium.

All previously published mutants for which the protease activity has been assessed and the four novel missense mutants reported here share a loss of catalytic activity, even though many of the

missense mutations do not target the SP domain. The inactivating mechanisms resulting from the mutations seem to be diverse, and may fall into different categories. In some cases the mutated protein is not targeted correctly to the plasma membrane and is retained in the endoplasmic reticulum or Golgi apparatus (Silvestri, et al., 2009). In other cases, the mutation directly modifies the active site of the protease (Silvestri, et al., 2008) and in several cases, the mutation prevents the activation of the zymogen into the active form of the enzyme without directly modifying the amino acid sequence of the SP domain. The reason for the repressive effect of many mutants with protease activity defects remains unclear, and is probably artifactual. These observations suggest that the findings from the repression assay used to assess the functional relevance of MT2 protein variants should be interpreted with caution and may be misleading. In practice, we suggest that SP activity in the cell medium of transfected cells should be measured directly using the chromogenic substrate. This is a simple *in vitro* test used to determine whether a sequence variation leading to an amino acid substitution is functionally relevant or not.

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Figure legends

Figure 1: Location of the mutations in the MT2 protein.

- A. Schematic representation of the MT2 domains and location of the various mutations studied. The autoactivating cleavage site in the consensus RIVGG sequence is indicated by an arrowhead. L= LDLRA domain, TM= transmembrane domain.
- B. MT2 protein sequences from different species were aligned using the blastp software from the NCBI server. Sequences were from the following GenBank entries: Homo sapiens: NP_998818.1, Canis lupus: XP_001154526.1, Mus musculus: XP_533038.2, Rattus norvegicus:

XP_589838.2, Gallus gallus: NP_081402.2. Amino acids mutated in IRIDA patients in this study are outlined.

Figure 1: Location of the mutations in the MT2 protein

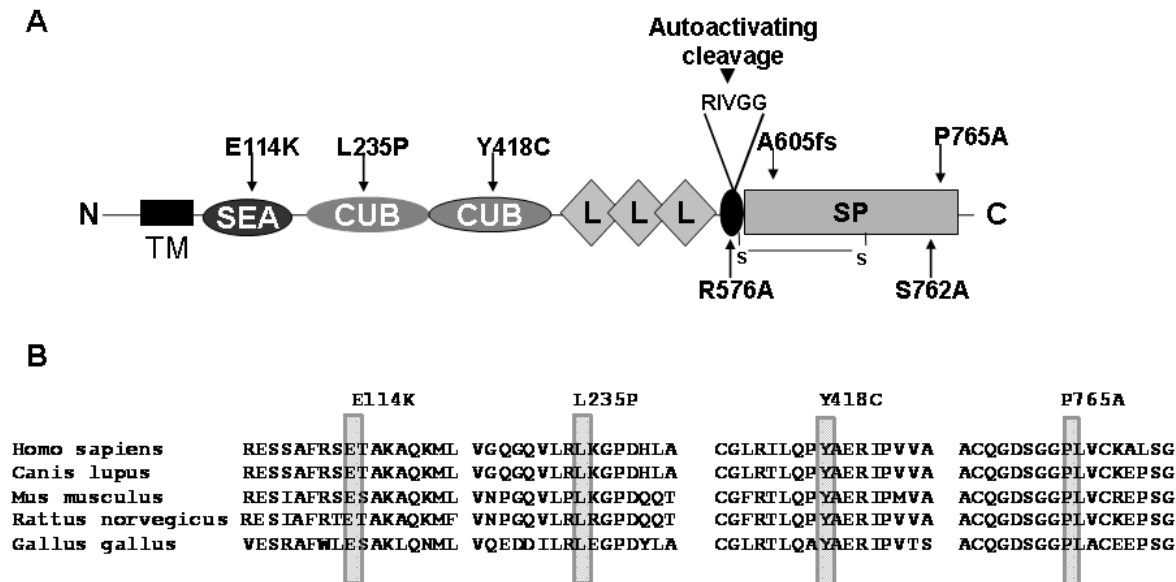


Figure 2: Family pedigree of the IRIDA patients and *TMPRSS6* genotypes.

Figure 2: Family pedigree of the 5 IRIDA patients and TMPRSS6 genotypes

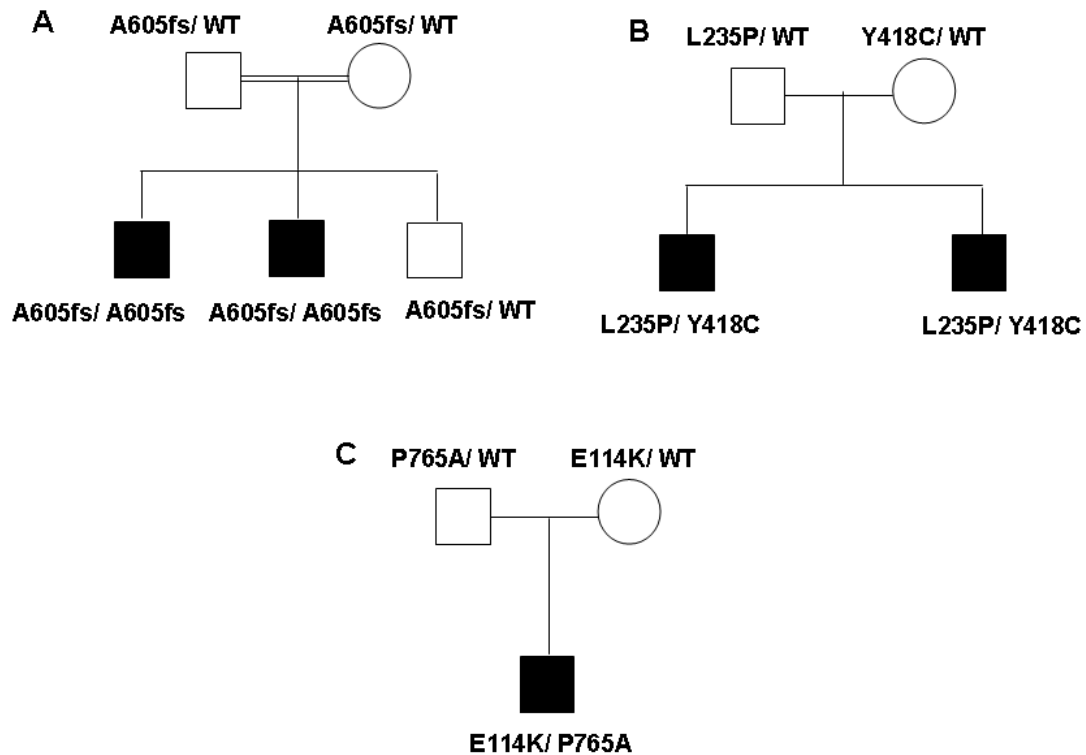


Figure 3: Repression of the *HAMP* promoter by MT2 variants.

Huh7 cells were transfected with the *HAMP* promoter/luciferase construct, with TK renilla as control. They were also co-transfected with an HJV-expressing vector alone (mock), or HJV plus an MT2-expressing vector: WT MT2, Y418C MT2, L235P MT2, E114K MT2, P765A MT2, R567A MT2, S762A MT2, or A605fs MT2. Relative luciferase activity was determined as the ratio of luciferase/renilla activity. Values indicate mean activity and error bars indicate the SD. P values were calculated using Student's t-test. §= *HAMP* promoter activity significantly different from the activity generated by HJV alone ($P < 0.05$), *= *HAMP* promoter repression significantly different from WT MT2 repression ($P < 0.05$). Each transfection was done in triplicate.

Figure 3 : Repression of *HAMP* promoter by MT2 mutants

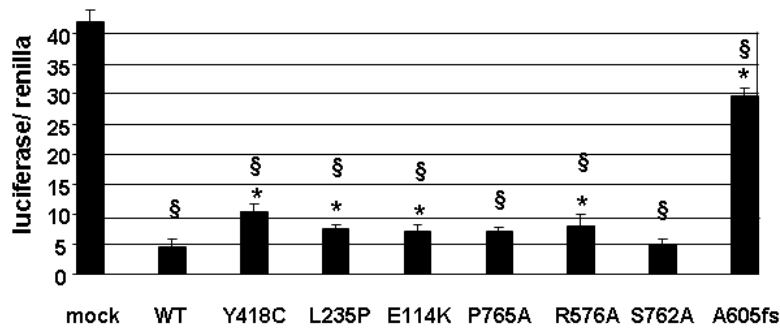


Figure 4: Autoactivation of MT2 variants.

HeLa (A) and Huh7 (B) cells were transiently transfected with HJV-expressing vector alone (mock), HJV and either WT, or Y418C, L235P, P765A, E114K, R567A, S762A mutant MT2-FLAG. HeLa cells were also transfected with the A605fs mutant. Whole cell extracts and concentrated media were loaded onto 10% and 12% SDS PAGE, respectively, transferred onto the membrane, and hybridized with a monoclonal anti-FLAG primary antibody for MT2 detection. Loading controls in cell lysates were revealed by anti-actin antibodies.

The scale shows the molecular mass in kiloDaltons. CL= cell lysates, CM= concentrated media, SP=serine protease domain. Arrows indicate full length or A605fs MT2.

Figure 4: Defective autoactivation of MT2 IRIDA variants

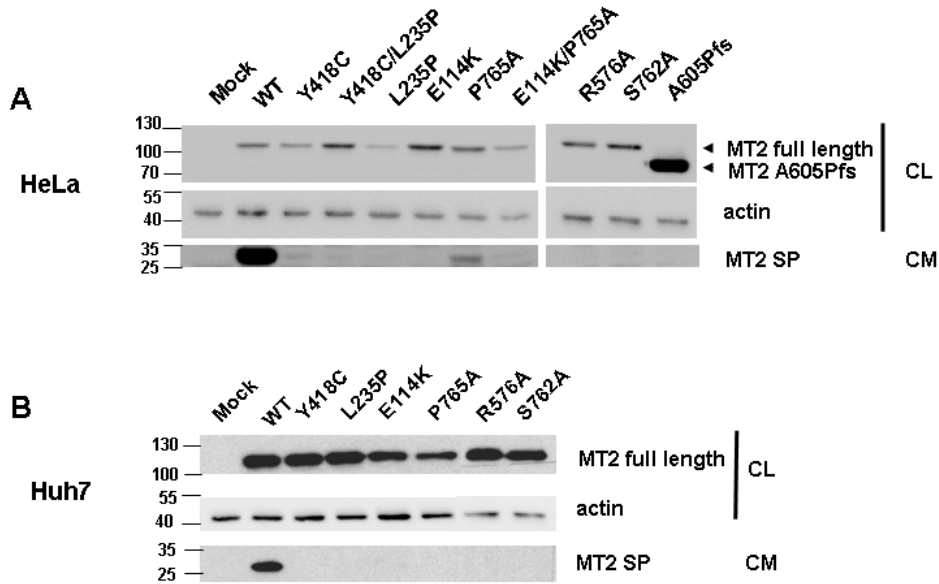


Figure 5: The four missense IRIDA mutants are not transactivated by WT MT2.

Huh7 cells were transfected with an HJV expressing vector. They were also transfected with two additional plasmids, one being either the empty vector or WT MT2-V5, and the other a mutated MT2-FLAG. Cell lysates (CL) and concentrated media (CM) were loaded onto 10% or 12% SDS PAGE respectively. Membranes were probed with anti-FLAG antibodies for the detection of MT2 mutants, and with anti-V5 antibodies for the detection of WT MT2-V5.

Figure 5: The 4 missense IRIDA mutants are not transactivated by wt MT2

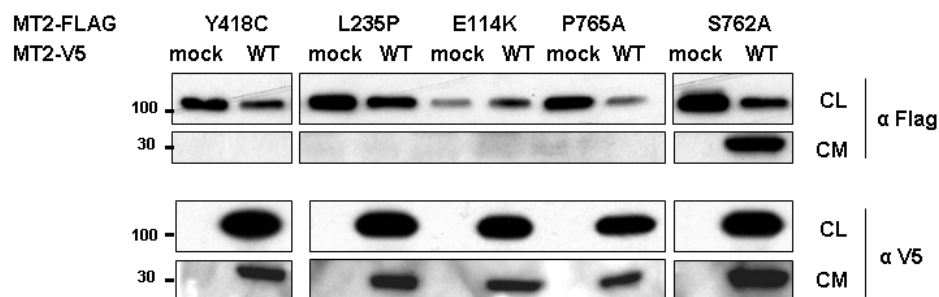
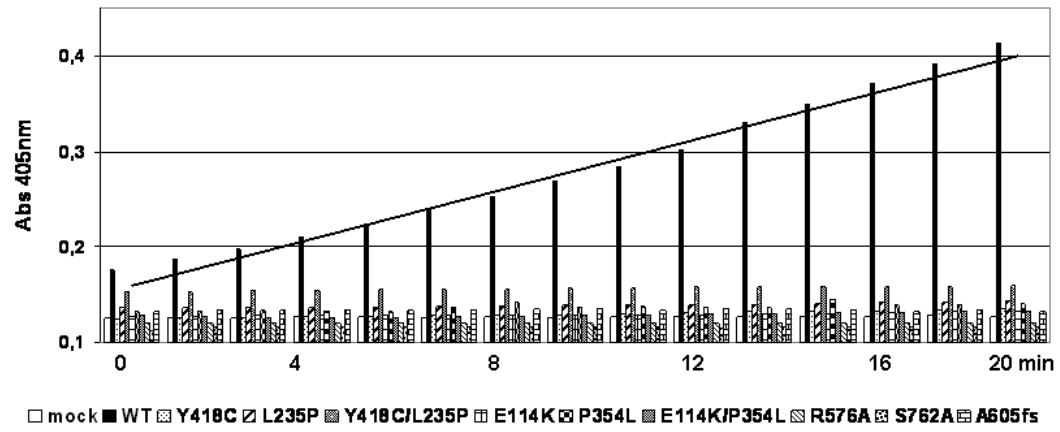


Figure 6: The medium from cells transfected with IRIDA mutants was proteolytically inactive. HeLa cells were transfected with HJV and MT2-expressing constructs. Activity was assayed at 37°C, for concentrated medium (15 μ g of proteins) from transfected HeLa cells, by monitoring the release of p-nitroanilide from the chromogenic substrate N-(tert-butoxycarbonyl)-Gln-Ala-Arg-p-nitroanilide at a wavelength of 405 nm, for up to 20 minutes.

Figure 6: MT2 mutants are proteolytically inactive



UNCORRECTED

Table 1: *TMPRSS6* mutations and biological parameters of IRIDA patients

| family | <i>TMPRSS6</i> genotype | amino acid change | Data at diagnosis | | | Current data | | | | | |
|----------|----------------------------|----------------------|-------------------|--------------|-------------|---------------|------------------------------|--------------------|-----------|--------------|-------------|
| | | | age (year) | Hb (g/dL) | MCV (fL) | Age (year) | serum hepcidin (ng/mL) | ferritin (µg/l) | TSAT % | Hb (g/dL) | MCV (fL) |
| A | c.[1813 delG]; | p.[A605PfsX8]; | 15 | 7.3 | 54 | 25 | 4288 | 230 | 4 | 12 | 64 |
| | [1813 delG] | [A605PfsX8] | 1 | 7.4 | 51 | 17 | ND | 66 | 6 | 12 | 71 |
| B | c.[1253A>G]; | p.[L235P]; | 4 | 7.7 | | 11 | 62 | 90 | 5 | 10.5 | 64 |
| | [704 T>C] | [Y418C] | 0.5 | 9 | | 8 | 81 | 115 | 3 | 9.6 | 70 |
| C | c.[340 G>A]; | p.[E114K]; | 3 | 10.2 | 61 | 7 | 59 | 20 | 4 | 10.8 | 71 |
| | [2293 C>G] | [P765A] | | | | | | | | | |

Hematological parameters and parameters of iron status before (at diagnosis) or after (current data) iron treatment, and *TMPRSS6* genotypes in the 5 patients from 3 families.

Hb= Hemoglobin concentration, reference range: 13.5-17.5 (males), 12.5-15.5 (females). MCV= Mean Cellular Volume, reference range: 80-99 fL. Heparidin concentration, reference range: 29-254 (males), 16-288 (females) (Ganz, et al., 2008). Ferritin concentration, reference range: 30-300 (males), 20-200 (females). TSAT= Transferrin Saturation, reference range: 20–40% (males), 15–35% (females).

Table 2: Functional studies of MT2 mutations published in the literature

| MT2 Mutation | Autocleavage | Repression of HJV-induced <i>HAMP</i> promoter activity | | Reference |
|---------------------------|--------------|---|-----------------------------|---------------------------|
| | | ≠ between mock and MT2 mutant | ≠ between WT and mutant MT2 | |
| | | | | |
| A118D | No | Yes | Yes | (Ramsay, et al., 2009b) |
| P686fs | No | Yes | Yes | (Ramsay, et al., 2009b) |
| I212T | Weak | Yes | Yes | (De Falco, et al., 2010) |
| R271Q | Yes | Yes | No | (De Falco, et al., 2010) |
| G442R | Weak | Yes | Yes | (Silvestri, et al., 2009) |
| D521N, E522K | No | Yes | Yes | (Silvestri, et al., 2009) |
| MT2^{MASK} | ND | No | Yes | (Silvestri, et al., 2008) |
| Y141C | No | Yes | Yes | (Altamura, et al., 2010) |
| R774C | No | Yes | Yes | (Silvestri, et al., 2008) |
| Y418C | No | Yes | Yes | This paper |
| L235P | No | Yes | Yes | This paper |
| E114K | No | Yes | Yes | This paper |
| P765A | Weak | Yes | No | This paper |
| A605fs | No | Weak | Yes | This paper |

Summary of the results obtained for autocleavage of MT2 and *HAMP* repression in the luciferase assay, from different papers describing MT2 mutations, and from this paper. ND = Not Determined